Expressed Sequence Tag Analysis of *Eimeria*-Stimulated Intestinal Intraepithelial Lymphocytes in Chickens

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Abstract

Intraepithelial lymphocytes (IELs) play a critical role in protective immune response to intestinal pathogens such as *Eimeria*, the etiologic agent of avian coccidiosis. A list of genes expressed by intestinal IELs of *Eimeria*-infected chickens was compiled using the expressed sequence tag (EST) strategy. The 14,409 ESTs consisted of 1851 clusters and 7595 singletons, which revealed 9446 unique genes in the data set. Comparison of the sequence data with chicken DNA sequences in GenBank identified 125 novel clones. This EST library will provide a valuable resource for profiling global gene expression in normal and pathogen-infected chickens and identifying additional unique immune-related genes.

Index Entries: Chickens; intestinal lymphocytes; expressed sequence tag (EST); expression cDNA library; *Eimeria.*

1. Introduction

Mucous membranes lining the gastrointestinal tract are the major sites of entry for many infectious agents. Intestinal mucosal surfaces are defended against pathogens by a group of organized gut-associated lymphoid tissues (GALT). The primary effector cells of the GALT are intraepithelial lymphocytes (IELs) localized in the outer mucosal epithelial layer *(1)*. The IELs recognize and destroy pathogens that breach the intestinal epithelium. Chicken intestinal IELs are composed of two phenotypically and functionally distinct subpopulations, natural-killer (NK) cells and T-lymphocytes *(2)*. Both cell types are the major effectors against coccidia parasites, intracellular protozoa belonging to the genus *Eimeria*. Avian coccidiosis is the most economically important disease for the poultry industry and considerable research efforts have been directed at identifying the protective immune mechanisms at the cellular and molecular levels *(1,3–7)*. Toward this end, cellular immunity mediated by intestinal IELs and their cytokines have been shown to confer protective immunity in response to *Eimeria* infection *(3–5)*. However, progress in this area has been historically slow, mainly limited by the lack of sequence homology between mammal and avian that hinders cloning and characterization of chicken immune-regulated genes by classic techniques of molecular biology *(8)*. In an attempt to circumvent this problem, we prepared a cDNA library from intestinal IELs of *Eimeria*-infected chickens and

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used expressed sequence tag (EST) technology to identify genes encoding proteins involved in the protective immune response.

2. Materials and Methods

2.1. Animals, Parasites, and Experimental Infections

Fertilized eggs of White Leghorn SC chickens were purchased from HyVac (Adel, IA) and hatched at the Animal and Natural Resources Institute (Beltsville, MD). Chickens were given unlimited access to feed and water and constant light was provided during the entire experimental period. Wild-type strains of *E. acervulina* and *E. maxima* were cleaned by flotation on 5.25% sodium hypochlorite and washed three times with Hanks' balanced salt solution (HBSS, Sigma, St. Louis, MO). Chickens ($N = 5$) were orally infected at 3 wk of age with 1×10^4 sporulated oocysts and at 9 wk of age with 2×10^4 sporulated oocysts of the homologous parasite.

2.2. Construction and Analysis of an IEL cDNA Library

Intestinal IELs were collected from the duodenum (for *E. acervulina*) or between the Meckel's diverticulum and the ileac region (for *E. maxima*) at 4 d after primary and secondary infections as described ***(9)***. Tissues were pooled from five chickens, washed with ice-cold Ca^{2+}- and Mg^{2+}-free HBSS (CMF-HBSS) containing 1 m*M* dithiothreitol (DTT, Sigma), cut into 3-cm sections, and incubated in CMF-HBSS containing 0.5 m*M* EDTA and 5% fetal calf serum (FCS) for 20 min at 37°C with constant swirling. Cells released into the supernatant were pooled, passed through nylon wool (Robbins Scientific, Sunnyvale, CA), and washed twice with CMF-HBSS containing 5% FCS. The IELs were purified on a discontinuous Percoll density gradient (Sigma) by centrifugation at 600*g* for 25 min at 24°C and washed three times with CMF-HBSS containing 5% FCS. Cell viability was consistently greater than 95% and lymphocytes were more than 80% as determined by trypan blue dye exclusion assay. Using flow cytometry (EPICS-XL, Coulter, Hialeah, FL), IELs were composed of 21% $CD3^+$, 70% $CD8^+$, and 9% $CD4^+$ lymphocytes. Total RNA was isolated using TRIzol (Life Technologies, Gaithersburg, MD) and used for purification of mRNA, cDNA synthesis, and library construction. We used a proprietary nucleic acid subtraction-based approach for normalization (Life Technologies, US Patent No. 60/059,817) where a directionally cloned cDNA library was depleted of its most abundant components by self-subtracting with biotinylated driver RNA. The cDNA inserts were directionally cloned into the *Sal*I/*Not*I sites of the pCMV-SPORT 6 vector and transformed into *Escherichia coli* EMDH10B. Individual clones were picked, inoculated into 384-well plates, and cultured overnight in LB broth. Polymerase chain reaction (PCR)-based 5'-end single-pass DNA sequence analysis was performed as described ***(10)*** on an ABI 3700 (Applied Biosystems, Forster City, CA). The ESTs consisting of vector sequences, sequences shorter than 100 bp and ambiguous regions were eliminated from data analysis. Sequences greater than 100 nucleotides were compared against GenBank using the BLASTN and BLASTX programs and deposited in the GenBank dbEST database (accession numbers: CD726833–CD740810, and CF074760–CF075157).

3. Results and Discussion

This library contained 1.87×10^7 transformants with an average insert length of 1.72 kb (range, 0.5–3.0 kb). Individual clones (34,078) were randomly picked and sequenced generating 14,409 chicken-specific ESTs that could be grouped into 9446 unique contigs. The majority of contigs (7595; 80.4%) consisted of single ESTs and the remaining 1851 contigs were composed of clusters of two or more overlapping/identical ESTs (average of 3.7 ESTs per cluster contig). Most cluster contigs (1567; 84.7%) contained two to four ESTs comprising 30.6% (4418) of the total number of ESTs (**Fig. 1**). The contig average readable sequence length was 418 bp with almost half (45.8%) falling within the range of 400–600 bp (**Fig. 2**). The average sequence length per contig was 712 bp and 71.2% (1318) of all contigs ranged from 0.5 to 1.0 kb. The 7595 singleton ESTs represented more than half (52.7%) of the

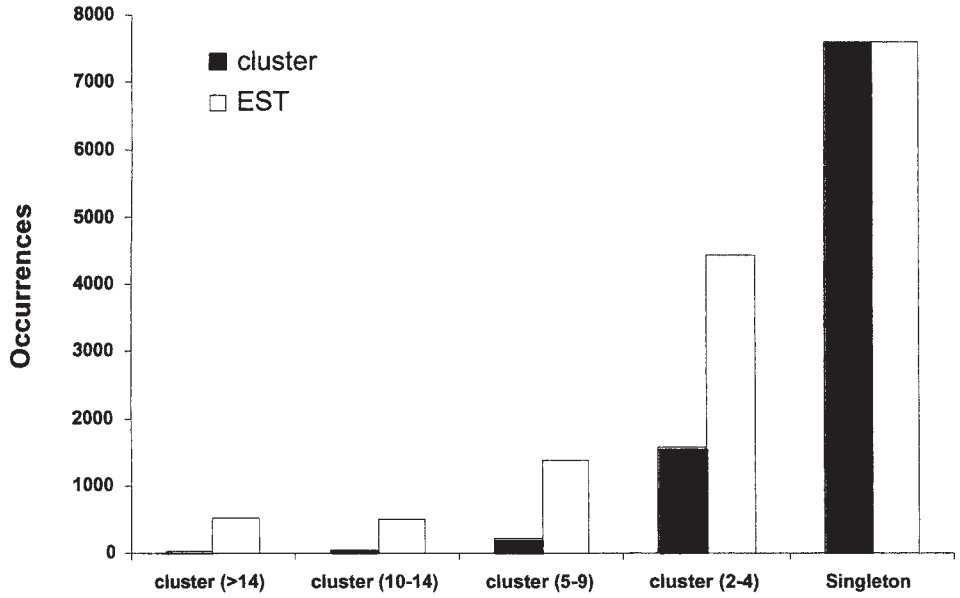

Fig. 1. Prevalence distribution of the cluster size. The 14,409 ESTs were grouped into 9446 cluster contigs consisting of 18 clusters (518 ESTs) with more than 14 ESTs, 42 clusters (502 ESTs) of 10–14 ESTs, 224 clusters (1376 ESTs) of 5–9 ESTs, 1567 clusters (4418 ESTs) of 2–4 ESTs, and 7595 singleton sequences. Most ESTs belonged to small-sized clusters and singletons indicating high complexity of the library.

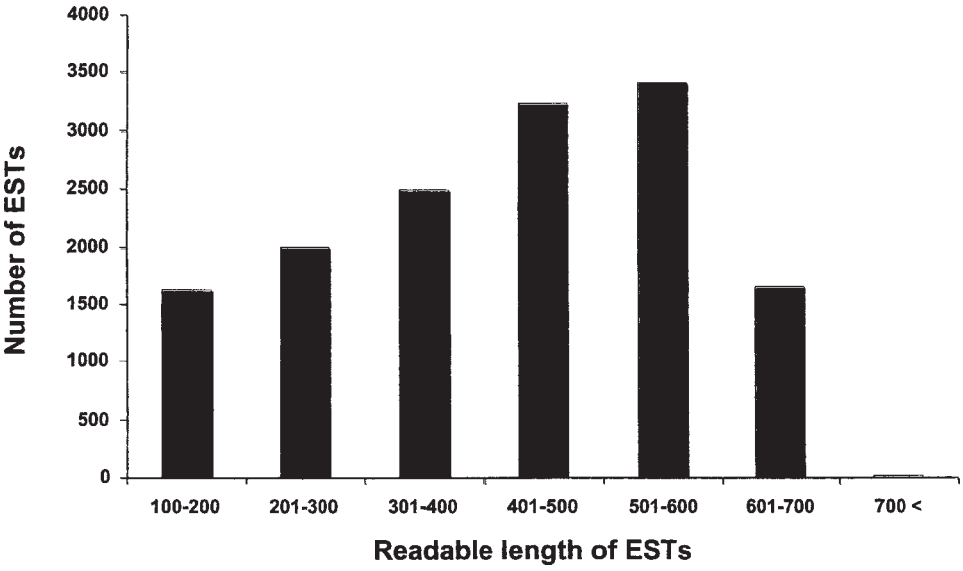

Fig. 2. Distribution of the readable length of ESTs. A total of 14,409 ESTs were generated in the present study. After elimination of vector and bacteria sequences and sequences shorter than 100 bases, the average of the readable sequence lengths was 418 bp with the major fraction of ESTs between 400 and 600 bp.

Table 1
Clusters That Contain More Than 14 ESTs

Contig ID	Gene description	Organism	Accession No.	No. of ESTs
Contig171	NK-lysin	Equus caballus	CD728315	87
Contig1648	Apolipoprotein AIV	Gallus gallus	CD731936	69
Contig42	Fatty acid binding protein	Gallus gallus	CD735219	51
Contig1279	Immunoglobulin α heavy chain	Gallus gallus	CD735924	43
Contig1234	2',5'-oligoadenylate synthetase	Gallus gallus	CD730844	24
Contig944	ATP synthase β-subunit	Cyprinus carpio	CD732620	24
Contig971	Interferon regulatory factor 6	Ovis aries	CD732407	22
Contig1300	Jun-binding protein	Gallus gallus	CD739778	20
Contig1325	Acidic ribosomal phosphoprotein (P0)	Gallus gallus	CD737516	20
Contig1000	Angiotensin converting enzyme	Gallus gallus	CD731489	19
Contig608	α-tubulin	Gallus gallus	CD736033	19
Contig1524	34/67 kDa laminin receptor	Cricetulus griseus	CD737204	18
Contig1792	Actin related protein 2/3 complex, subunit 1B (ARPC1B)	Homo sapiens	CD737537	18
Contig733	GAPDH	Gallus gallus	CD735039	18
Contig992	Unknown	Unknown	CD729072	18
Contig528	Na^+-dependent nucleoside transporter	Oryctolagus cuniculus	CD737431	17
Contig352	Unknown	Unknown	CD733292	16
Contig1247	Ferritin heavy chain	Gallus gallus	CD740150	15

total number of ESTs. In comparison, previous chicken cDNA libraries contained 31.4% ***(11)*** and 42.0% ***(12)*** single EST contigs. We suspect the higher percentage of unique sequences that we observed in our intestinal cDNA library was the consequence of two effects, use of a normalized cDNA library and inoculation with two *Eimeria* species, thereby expanding the number of induced immune response transcripts. However, there is a possibility that expression of some transcripts could have been affected by the IEL purification process.

To identify the sequences present in our cDNA library, we used the Basic Local Alignment Search Tool (BLAST) program ***(13,14)*** to perform sequence similarity searches against the GenBank nucleic acid sequence database. Initially, we analyzed the 18 cluster contigs containing 15 or more ESTs (**Table 1**), which could be regarded as abundant transcripts and therefore were most likely to match previously described genes. This group of contigs constituted 1.0% of the total number of clusters and 3.6% of the total ESTs. Ten sequences were highly similar to previously described chicken genes. Of them, apolipoprotein AIV, fatty acid-binding protein, acid ribosomal phosphoprotein, α-tublin, GAPDH, and ferritin heavy chain are highly expressed in other chicken tissues ***(11,15)***. Six contigs were similar to genes from other organisms (NK-lysin, ATP synthase beta-subunit, interferon regulatory factor 6, 34/67 kDa laminin receptor, actin-related protein 2/3 complex, subunit 1B, and Na+-dependent nucleoside transporter). The remaining two sequences did not display similarities to any known genes in the database.

Interestingly, contig171, composed of 87 ESTs, occurred with the highest prevalence in this *Eimeria*-induced intestinal cDNA library. By BLAST searching, this gene was weakly similar to NK-lysin or granulysin (BLASTX score = 45) of pig, horse, and human origins. Sequence analysis indicated that contig171 contains a 420-bp open reading frame predicted to encode a protein of 140 amino acids with molecular mass of 15.2 kDa with SapB domain (E-value = 6.15^{-14}). This indicated that contig171 is the avian homolog of NK-lysin. Low levels of NK-lysin mRNA were detected

Table 2
Gene Description of EST Clones Encoding Novel Genes

Accession No.	Gene description	Organism
CD738626	Cell adhesion receptor CD36	Homo sapiens
CD733649	C-C chemokine receptor type 8	Homo sapiens
CD731058	Unc-51-like kinase 1	Homo sapiens
CD728387	Death-associated protein kinase 2	Homo sapiens
CD735090	Molybdenum cofactor sulfurase	Homo sapiens
CD738954	Lysosomal α-glucosidase precursor	Homo sapiens
CD729517	FYVE and coiled-coil domain containing 1	Homo sapiens
CD738748	NADH-ubiquinone oxidoreductase 23 kDa subunit	Homo sapiens
CD736957	Cytochrome b	Homo sapiens
CD736420	Proline rich protein 2	Mus musculus
CD735751	XE7 protein	Homo sapiens
CD730299	Glypican-5 precursor	Homo sapiens
CD729519	Nuclear receptor ROR gamma	Homo sapiens
CD727541	LPS-induced TNF-α factor	Mus musculus
CD736226	Chicken interleukin-17	Gallus gallus
CD734961, CD730399, CD734119, CD728379, CD727597, CD727400, CD731169, CD731115, CD730361, CD740652, CD740042, CD736629, CD732509, CD728248, CD733849, CD736417, CD733039, CD732261, CD729400, CD727352,CD739375, CD739243, CD738814, CD738713, CD737546, CD737436, CD737265, CD737143, CD736753, CD736574, CD735803, CD735525, CD735034, CD734634, CD734415, CD734205, CD734174, CD731039, CD732864, CD732742, CD732533, CD732483, CD732046, CD731574, CD731464, CD731383, CD731015, CD730413, CD730405, CD729425, CD729336, CD729146, CD728815, CD728724, CD727914, CD727364, CD727346, CD727325, CD727314, CD740498, CD739797, CD739792, CD739398, CD739228, CD739207, CD738908, CD738767, CD738387, CD737500, CD737318, CD737058, CD736502, CD736237, CD735412, CD735331, CD735269, CD734571, CD734450, CD734290, CD734232, CD733879, CD733169, CD732522, CD731579, CD731230, CD731160, CD731030, CD730798, CD730781, CD730438, CD730130, CD729847, CD729580, CD729560, CD729479, CD728607, CD728459, CD728329, CD728298, CD727732, CD727530, CD727390, CD727381, CD727362, CD727321, CD727277, CD727250, CD727130, CD726977, CD726937	Unknown	

in tissues known to contain NK and T-cells and increased gene expression was observed when a lymphocyte fraction enriched in NK and T-cells was stimulated by IL-2 ***(16,17)***. Similarly, the IELs used in this study contained a high percentage of NK and T-cells (data not shown) and were stimulated by *Eimeria* antigen. Furthermore, we recently showed that the gene corresponding to contig171 was significantly upregulated after primary and secondary infections with *E. acervulina* and *E. maxima* ***(7)***.

Another highly prevalent EST in our data set is 2' ,5'-oligoadenylate synthetase, an enzyme induced by interferon (IFN)-treated chicken embryo cells ***(18)***. This enzyme activity was increased by CpG-oligodeoxynucleotide stimulation of ovine peripheral blood mononuclear cells ***(19)***. Brown et al. ***(20)*** demonstrated that DNA from the protozoan parasite *Babesia bovis* contained CpG immunostimulatory sequences and showed mitogenic properties for mammalian leukocytes. We previously showed increased chicken IFN-γ levels after infection with *Eimeria* ***(5,7)***; however, whether or not *Eimeria* contains CpG immunostimulatory sequences remains to be investigated.

One of the principal aims of this cDNA cloning strategy was to identify chicken cytokine genes, as classic cloning procedures based on sequence similarities to mammalian cytokines have proven largely unsuccessful. With the recent availability of a large number of chicken EST clones derived from various tissues and whole chicken embryos ***(11,12,15,21)***, it is now possible to isolate and identify chicken cytokine genes as has been accomplished with IL-15 and IL-18 ***(22,23)***. During the course of characterizing our cDNA library, a chicken cytokine gene previously unidentified was sequenced and characterized as IL-17 ***(24)***.

Comparison of our intestinal cDNA sequence data with chicken DNA sequences in the GenBank identified 125 clones which encode novel genes (**Table 2**). Of 125 novel genes, 110 genes were unknown and the remaining genes showed weak similarities to C-C chemokine receptor type 8, cell adhesion receptor CD36, unc-51-like kinase 1, death-associated protein kinase 2, molybdenum cofactor sulfurase, lysosomal alpha-glucosidase precursor, FYVE and coiled-coil domain containing 1, NADH-ubiquinone oxidoreductase 23-kDa subunit, cytochrome b, proline-rich protein 2, XE7 protein, glypican-5 precursor, nuclear receptor ROR γ, and LPS-induced TNF-α factor.

In conclusion, we have used single-pass sequencing of *Eimeria*-stimulated intestinal IEL transcripts to study global gene expression profiling and to identify novel immune-related genes during avian coccidiosis. This approach led to the identification of the complete sequences of IL-16 and IL-17. Comparison of the current IEL EST data set with the database of Cogburn et al. *(15)* revealed 125 clones encoding novel transcripts indicating the existence of additional uncharacterized genes that should prove useful for future studies of the immune mechanisms to enteric pathogens such as *Eimeria* and *salmonella*.

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References

1. Neutra, N. R., Mantis, N. J., and Kraehenbuhl, J. P. (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. *Nat. Immunol.* **2,** 1004–1009.
2. Gobel, T. W., Kaspers, B., and Stangassinger, M. (2001) NK and T-cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. *Int. Immunol.* **13,** 757–762.
3. Lillehoj, H. S. and Trout, J. M. (1996) Avian gut-associated lymphoid tissues and intestinal immune responses to *Eimeria* parasites. *Clin. Microbiol. Rev.* **9,** 349–360.
4. Lillehoj, H. S. and Lillehoj, E. P. (2000) Avian coccidiosis. A review of acquired intestinal immunity and vaccination strategies. *Avian Dis.* **44,** 408–425.
5. Yun, C. H., Lillehoj, H. S., and Choi, K. D. (2000) *Eimeria tenella* infection induces local gamma interferon production and intestinal lymphocyte subpopulation changes. *Infect. Immunol.* **68,** 1282–1288.
6. Laurent, F., Mancassola, R., Lacroix, S., Menezes, R., and Naciri, M. (2001) Analysis of chicken mucosal immune response to *Eimeria tenella* and *Eimeria*

maxima infection by quantitative reverse transcription-PCR. *Infect. Immunol.* **69,** 2527–2534.
7. Min, W., Lillehoj, H. S., Kim, S., et al. (2003) Profiling local gene expression changes associated with *Eimeria maxima* and *Eimeria acervulina* using cDNA microarray. *Appl. Microbiol. Biotechnol.* **62,** 392–399.
8. Staeheli, P., Puehler, F., Schneider, K. Gobel, T. W., and Kaspers, B. (2001) Cytokines of birds: conserved functions—a largely different look. *J. Interferon Cytokine Res.* **21,** 993–1010.
9. Min, W., Lillehoj, H. S., Burnside, J., Weining, K. C., Staeheli, P., and Zhu, J. J. (2001) Adjuvant effects of IL-1β, IL-2, IL-8, IL-15, IFN-α, IFN-γ, TGF-β4 and lymphotactin on DNA vaccination against *Eimeria acervulina*. *Vaccine* **20,** 267–274.
10. Smith, T. P., Godtel, R. A., and Lee, R. T. (2000) PCR-based setup for high-throughput cDNA library sequencing on the ABI 3700 automated DNA sequencer. *Biotechniques* **29,** 698–700.
11. Tirunagaru, V. G., Sofer, L., Cui, J., and Burnside, J. (2000) An expressed sequence tag database of T-cell-enriched activated chicken splenocytes: sequence analysis of 5251 clones. *Genomics* **66,** 144–151.
12. Boardman, P. E., Sanz-Ezquerro, J., Overton, I. M., et al. (2002) A comprehensive collection of chicken cDNAs. *Curr. Biol.* **12,** 1965–1969.
13. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215,** 403–410.
14. Altschul, S. F., Madden, T. L., Schaffer, A. A., et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25,** 3389–3402.
15. Cogburn, L. A., Wang, X., Carre, W., et al. (2003) Systems-wide chicken DNA microarrays, gene expression profiling, and discovery of functional genes. *Poult. Sci.* **82,** 939–951.
16. Andersson, M., Gunne, H., Agerberth, B., et al. (1995) NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. *EMBO J.* **14,** 1615–1625.
17. Andersson, M., Gunne, H., Agerberth, B., et al. (1996) NK-lysin, structure and function of a novel effector molecule of porcine T and NK cells. *Vet. Immunol. Immunopathol.* **54,** 123–126.
18. West, D. K. and Ball, L. A. (1982) Induction and maintenance of 2',5'-oligoadenylate synthetase in interferon-treated chicken embryo cells. *Mol. Cell. Biol.* **2,** 1436–1443.
19. Mena, A., Nichani, A. K., Popowych, Y., et al. (2003) Innate immune responses induced by CpG oligodeoxyribonucleotide stimulation of ovine blood mononuclear cells. *Immunology* **110,** 250–257.
20. Brown, W. C., Estes, D. M., Chantler, S. E., Kegerreis, K. A., and Suarez, C. E. (1998) DNA and a CpG oligonucleotide derived from *Babesia bovis* are mitogenic for bovine B cells. *Infect. Immunol.* **66,** 5423–5432.
21. Abdrakhmanov, I., Lodygin, D., Geroth, P., et al. (2000) A large database of chicken bursal ESTs as a resource for the analysis of vertebrate gene function. *Genome Res.* **10,** 2062–2069.
22. Schneider, K., Puehler, F., Baeuerle, D., et al. (2000) cDNA cloning of biologically active chicken interleukin-18. *J. Interferon Cytokine Res.* **20,** 879–883.
23. Lillehoj, H. S., Min, W., Choi, K. D., et al. (2001) Molecular, cellular, and functional characterization of chicken cytokines homologous to mammalian IL-15 and IL-2. *Vet. Immunol. Immunopathol.* **82,** 229–244.
24. Min, W. and Lillehoj, H. S. (2002) Isolation and characterization of chicken interleukin-17 cDNA. *J. Interferon Cytokine Res.* **22,** 1123–1128.